Supercritical Fluid Extraction of Volatile *N*-Nitrosamines in Fried Bacon and Its Drippings: Method Comparison

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_N_-Nitrosopyrrolidine (NPYR) and _N_-nitrosodimethylamine (NDMA), known animal carcinogens, are consistently formed in bacon during frying. As a result, commercial bacon has been subject to regulatory monitoring and compliance for the past 20 years to ensure that _N_-nitrosamines do not exceed the 10 ppb violative level. Currently, time-consuming distillation–solvent extraction and solid-phase extraction (SPE) methods are used for this purpose. With an emphasis on reducing solvent use, we investigated supercritical fluid extraction (SFE) using supercritical carbon dioxide (SC-CO_2) for isolation of volatile nitrosamines common to fried bacon. Eighteen fried bacon samples were analyzed for NPYR and NDMA by SFE, SPE, mineral oil distillation (MOD), and low-temperature vacuum distillation (LTVD) methods, using the same gas chromatographic-chemiluminescence detection (thermal energy analyzer) conditions. The range of values for SFE was 0.7 to 20.2 ppb for NPYR and none detected (ND) to 2.4 ppb for NDMA. Analysis of variance of the NPYR data showed a significant difference ($p < 0.05$) between SFE and SPE results and significant differences between these and those obtained by MOD and LTVD. Overall, SFE was superior to the other methods with the highest recoveries, best repeatability, rapidity of analysis, and solvent-sparing characteristics. Similar results were obtained for SFE after comparison with distillation and SPE methods for determining the same nitrosamines in fried bacon drippings.

Because of recent U.S. Environmental Protection Agency regulations (1), there is a strong incentive to reduce or replace organic solvents, particularly those containing halogens, used in residue analysis. These regulations are designed to reduce the use of solvents that are potentially harmful to the environment and to reduce costs of solvent disposal. Therefore, solvent-sparing analytical methods are needed. Most current methods require selective separation of analyte from the sample matrix by multiple sample preparation treatments, including homogenization, distillation, solvent extraction–partition, concentration, and other cleanup steps. These procedures are time-consuming and labor intensive and may result in some analyte loss. Supercritical fluid extraction (SFE) has the potential to effectively achieve selective extraction in a single step and to concentrate the analyte so that it is ready for instrumental analysis with a minimum amount of solvent.

Despite obvious advantages and the development of commercial SFE systems, methods using SFE techniques have not been widely adopted for trace levels of residues in foods, especially meat and meat products. In the field of nitrosamines, SFE using supercritical carbon dioxide (SC-CO_2) with 10% methanol and commercial instrumentation was used to extract nicotine-derived nitrosamines from tobacco and tobacco products (2, 3). SFE was successfully used with SC-CO_2 alone to recover 10 volatile aliphatic and alicyclic nitrosamines from frankfurters at the 20 ppb level (4). More recently, our laboratory used an SFE method to extract *N*-nitrosodibenzylamine (NDBzA) from hams, which had resulted from contact with rubber-containing elastic nettings, and compared it with a solvent extraction method (5). These *N*-nitroso compounds were isolated by a novel integral restrictor–collector assembly that reduced the path length between the heated micrometering valve and the collector, trapping the nitrosamines on a sorbent bed of a commercial solid-phase extraction (SPE) cartridge. This feature was described in extensive detail by Maxwell et al. (6) and incorporated in a currently available commercial SFE instrument. The present study reports the expanded use of SFE for extracting volatile *N*-nitrosamines from fried bacon and its drippings. The SFE results were compared with those from 3 other AOAC methods.

METHOD

Caution: *N*-Nitrosamines are potential carcinogens. Exercise care in handling these compounds.

Materials

(a) *Bacon samples.*—Commercial bacon was obtained from local retail outlets. The bacon was fried in a preheated Farberware electric frying pan for 6 min (3 min/side) at a cali-

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brated temperature of 177°C. The bacon drippings were collected in a 250 mL Erlenmeyer flask and frozen (–20°C) until analyzed. The fried bacon was ground through a 1/16 in. plate, and then thoroughly mixed. The comminuted sample was vacuum packaged and stored in a –20°C freezer until analyzed. Fried bacon, without drippings, was also obtained from the U.S. Department of Agriculture's (USDA's) Food Safety and Inspection Service (FSIS), Eastern Laboratory, Athens, GA.

(b) *Reagents.*—The sources and purification of the reagents used in the analysis of fried bacon by the 4 methods were described in detail elsewhere (7, 8). Morpholine was doubly distilled before use and then checked for the presence of *N*-nitrosomorpholine (NMOR) as a contaminant; none was found. 2,6-Dimethylmorpholine was free of the corresponding nitrosamine and was used without further purification. The silica gel (7734) used in SPE columns was from E. Merck (Cherry Hill, NJ). The 70–230 mesh (grade 60) material was washed twice with dichloromethane (DCM), filtered, and dried 4 h in a vacuum oven at 60°C. It was sieved to a particle range of 70–150 mesh before use. The sieved silica gel was packed into empty 6 mL SPE columns using frits provided by Applied Separations, Inc. (Allentown, PA).

(c) *N-Nitrosodipropylamine (NDPA) internal standard solution.*—0.10 μg/mL in DCM.

(d) *Gas chromatographic working standard solution.*—Each, 0.10 μg/mL in DCM: *N*-nitrosodimethylamine (NDMA), *N*-nitrosomethylethylamine (NMEA), *N*-nitrosodiethylamine (NDEA), NDPA, *N*-nitrosoazetidine (NAZET), *N*-nitrosodibutylamine (NDBA), *N*-nitrosopiperidine (NPIP), *N*-nitrosopyrrolidine (NPYR), NMOR, and *N*-nitrosohexamethyleneimine (NHMI). These nitrosamines were synthesized from their corresponding amines and sodium nitrite according to the general procedure published previously (9).

Apparatus

(a) *SFE system.*—The extractor was obtained commercially (Applied Separations, Inc.). The instrument was configured for parallel extraction of 2 SFE vessels and designed so that commercial 6 mL SPE cartridges (Applied Separations, Inc.) could be attached directly to the micrometering valves without the aid of fittings or connecting tubing. All components of this instrument are shown in Figure 1. A detailed description of the instrument was published elsewhere (5).

(b) *Gas chromatograph–Thermal Energy Analyzer (TEA).*—The instrument and the operating conditions for separation and quantitation of nitrosamines were described elsewhere (8).

Procedures

(a) *SFE.*—Complete details of this procedure were described previously (5). Briefly, weigh 5.0 g fried bacon into 100 mL beaker. Add 250 mg propyl gallate, fortify with 0.5 mL NDPA internal standard, add 5.0 g Hydromatrix (Varian-Analytichem, Harbor City, CA), and stir mixture with glass rod until uniform. Transfer mixture to extraction vessel; install extraction vessel in SFE system. Preheat micrometering valves to 115°C; set SFE oven to 40°C. Attach 6.0 mL SPE cartridge containing 1.0 g silica gel to micrometering valves. Extract at 10 000 psi (680 bar) with flow rate of expanded CO_2 gas of 2.8 L/min for a total of 50 L. Wash SPE cartridges with 8.0 mL pentane–DCM (75 + 25) and elute nitrosamines with 8.0 mL DCM–ether (70 + 30). Concentrate to 1.0 mL and quantitate nitrosamines on GC–TEA for this procedure and those described in sections **(b)** through **(g)**.

(b) *SPE.*—The procedure for analysis of fried bacon by SPE is the same as that described elsewhere for ham (8). Briefly, weigh 10.0 g fried bacon into glass mortar. Add 250 mg propyl gallate, fortify with 1.0 mL NDPA internal standard, add anhydrous sodium sulfate and Celite, and then grind mixture until uniform. Transfer free-flowing mixture to a glass chromatographic column (32 × 400 mm) and elute nitrosamines with DCM, collecting DCM in Kuderna-Danish (K-D) apparatus. Concentrate eluate and transfer to silica gel solid-phase cartridge. Wash with pentane–DCM and elute nitrosamines with DCM–ether.

(c) *Low-temperature vacuum distillation (LTVD).*—Samples were analyzed by a procedure developed by Sen et al. (10) and described in detail in the *FSIS Chemistry Laboratory Guidebook* (11). Briefly, distill 10.0 g fried bacon, without any nitrosation inhibitors, at ca 45°C under vacuum (20–25 torr) from 200 mL 3N KOH. Acidify aqueous distillate and extract with DCM. Wash DCM with acid and base, dry with anhydrous sodium sulfate, and concentrate.

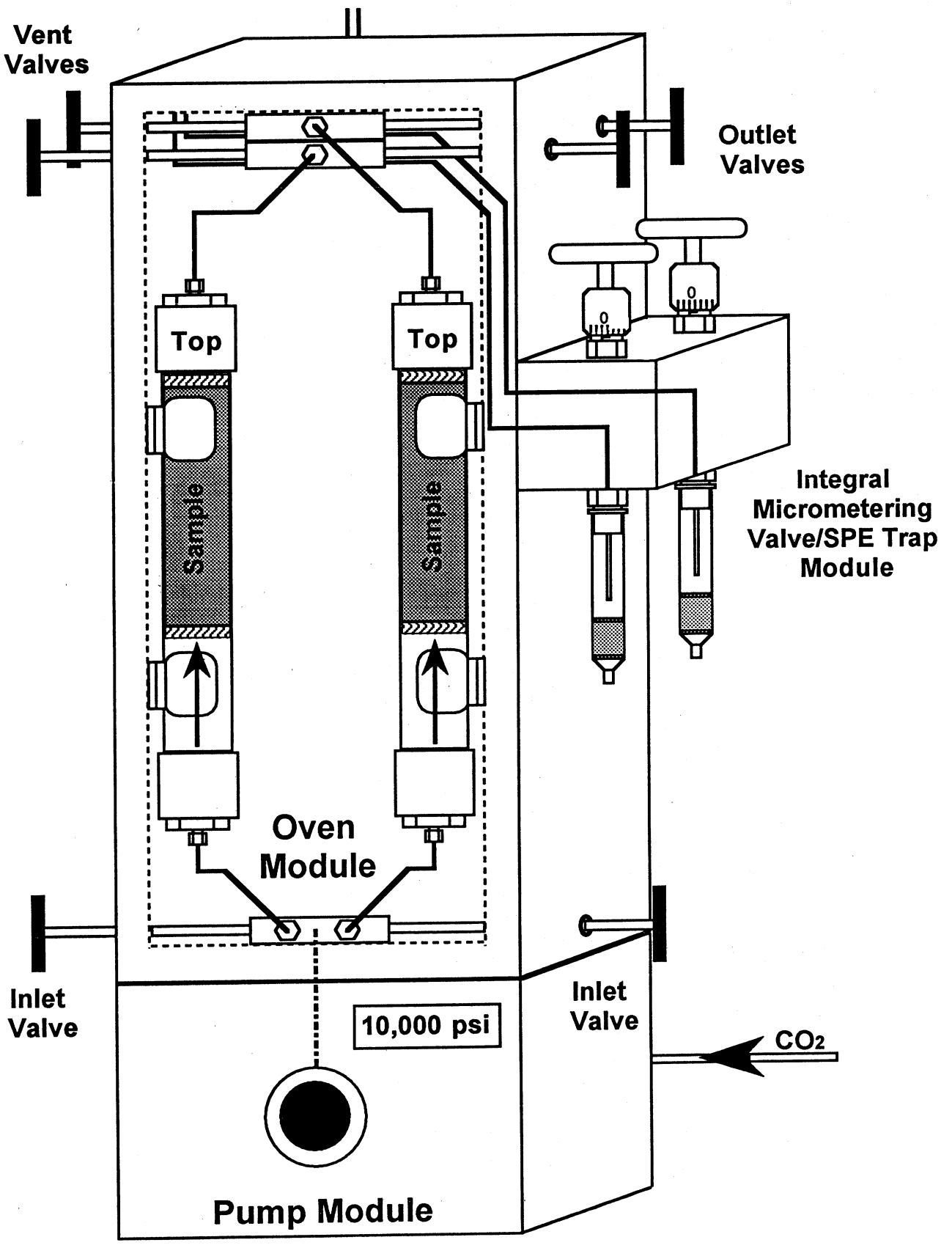
(d) *Mineral oil distillation (MOD).*—Samples were analyzed by a method originally developed by Fine et al. (12) and described in the *FSIS Chemistry Laboratory Guidebook* (11). Briefly, distill 10.0 g fried bacon, without any nitrosation inhibitors, under vacuum (<2 torr) from 2.0 mL 0.2N NaOH and 25 mL mineral oil. Extract aqueous distillate with DCM, dry, and concentrate.

(e) *Bacon drippings (distillation).*—Samples were analyzed by a method originally developed by White et al. (13). Briefly, distill 20.0 g drippings, solubilized with 50 mL DCM, from 80 mL 5N NaOH and 8.0 g barium hydroxide. Extract aqueous distillate with DCM, wash DCM with 50 mL 6N HCl and with 50 mL 5N NaOH, dry, and concentrate.

(f) *Bacon drippings (SPE).*—Weigh 2.5 g drippings into 50 mL beaker. Add 10.0 mL pentane and fortify with 0.5 mL NDPA internal standard. Prewash silica gel solid-phase cartridge with 30 mL pentane. When pentane reaches top of silica gel, add sample solution, rinse beaker 2 times with 10 mL pentane, and add rinses to column. Wash column with 30 mL pentane–DCM mixed solvent, and elute nitrosamines 2 times with 30 mL ether–DCM.

(g) *Bacon drippings (SFE).*—Weigh 2.5 g drippings into beaker containing 5.0 g Hydromatrix and 250 mg propyl gallate. Fortify with 0.5 mL NDPA internal standard. Mix thoroughly, add to SFE extraction vessel, and extract as described above for bacon. SFE operating conditions: flow rate, 2.2 L/min; total volume of expanded CO_2, 25 L; oven temperature, 40°C; micrometering valve, 115°C.

(h) *Nitrosamine quantitation.*—Complete details for the quantitation of volatile *N*-nitrosamines were published elsewhere (8). Values for NPYR, the predominant nitrosamine, in


Vent Valves
Outlet Valves
Top
Top
Sample
Sample
Integral Micrometering Valve/SPE Trap Module
Oven Module
Inlet Valve
10,000 psi
Inlet Valve
CO_2
Pump Module

the individual samples were corrected for recovery of NDPA internal standard. Minimum levels of reliable measurement (signal-to-noise ratio > 2) for 9 volatile nitrosamines were NDMA, NMEA, and NDEA, 0.2 ppb; NAZET, NPIP, NPYR, NMOR, and NHMI, 0.5 ppb; and NDBA, 1.0 ppb.

(i) *Statistical analysis.*—Data were analyzed by analysis of variance (ANOVA) and means procedures of the Statistical Analysis System PC software distributed by SAS Institute, Inc. (14). These results were then interpreted according to the methods of Snedecor and Cochran (15).

Results and Discussion

An initial problem encountered in the analysis of frankfurters by SC-CO_2 was artifactual formation of NDMA during the sample extraction step. This was demonstrated by its absence when other analytical methods are used and by consistent recoveries over 100% (4). It was verified by adding morpholine, a rapidly nitrosatable amine, to the sample before SFE, and detecting NMOR after extraction and cleanup. In this case, artifactual formation of nitrosamine was prevented by additing propyl gallate, a known nitrosation inhibitor, and by lowering the extraction temperature from 80° to 40°C. Use of the same SFE method and conditions for determining NDBzA in hams processed in elastic rubber netting also resulted in no artifactual nitrosamine formation (5).

Since the mid-1970s, fried bacon has been subject to regulatory monitoring and compliance for its nitrosamine content to ensure that no confirmable levels of nitrosamines (10 ppb) are present (16). As a result, we investigated whether SFE technology could also be applied to fried bacon and its drippings. We addressed the question of artifactual nitrosamine formation or false positives that would question the reliability of any method devised. Either morpholine or 2,6-dimethylmorpholine was added at the 1.0 ppm level to some of the fried bacon before SFE; none of the corresponding nitrosamines was detected.

During bacon extraction with SC-CO_2, the micrometering valve was set at 110°C, the same temperature previously used for frankfurter and ham extraction (4, 5). However, at this temperature, the discharge tube occasionally clogged with fat during the last 2–4 min of extraction, causing an increase in back pressure that blew the silica gel cartridge off the retaining nut. Increasing the micrometering valve temperature to 115°C to keep the fat liquefied eliminated this problem.

Recoveries of 10 volatile nitrosamines added to a nitrosamine-free, fried turkey bacon sample at the 10 ppb level and analyzed by SFE are shown in Table 1. Two of these volatile nitrosamines, NPYR and to a lesser extent NDMA, have been found consistently in cooked bacon and are not present in the uncooked product (17, 18). The mean recovery of NPYR was 104.1 ± 4.0% and of NDMA, 100.2 ± 11.6%. Mean recoveries for all other nitrosamines ranged from 87.3 to 108.0%. These results compare favorably with those reported for both frankfurters (4) and ham (5).

Eighteen duplicate samples of fried bacon were analyzed for NPYR and NDMA by the 4 isolation procedures (SFE, SPE, MOD, and LTVD) and the same GC–TEA detection conditions. The mean NPYR and NDMA results, corrected for recovery of the NDPA internal standard, are shown in Table 2. Highly significant ($p < 0.01$) differences were found among samples. An outlier test performed on the data indicated that no value or pair of values were outliers. Individual NPYR values ranged from 0.7 to 20.2 ppb for SFE, 0.6 to 18.8 ppb for SPE, 1.0 to 24.6 ppb for MOD, and 0.8 to 32.1 ppb for LTVD. Only 2 of the 18 samples of fried bacon exceeded the violative level of 10 ppb. The data were examined by ANOVA, and the means of the methods were further analyzed by Duncan's multiple range test at the $p < 0.05$ level (Table 3). The results showed no statistical difference in mean NPYR values between the MOD and LTVD methods. There was a significant difference between these methods and the SFE method, which differed significantly from the SPE method. The mean NPYR values for the distillation methods (MOD and LTVD), were significantly higher than those for the nondistillation methods (SFE and SPE). This finding suggests that the latter 2 methods are not as efficient in isolating NPYR from the sample matrix or that there is minor artifactual formation of NPYR during sample analysis by the MOD and LTVD methods. Average recoveries for the internal standard (NDPA) for each of the methods were SFE, 98.6 ± 5.9%; SPE, 95.9 ± 9.2%; MOD, 91.0 ± 12.8%; and LTVD, 84.0 ± 7.7%.

Statistical analysis of these recoveries showed that SFE and SPE were not significantly different ($p < 0.05$) from each other but were significantly different from other methods. This finding suggested that nondistillation methods might be more effective in isolating NPYR from fried bacon and that artifact formation was possible for distillation methods. Neither distillation method uses nitrosation inhibitors during analysis other than strong alkali, whereas, propyl gallate is added in the SFE and SPE methods to prevent artifactual formation caused by simultaneous presence of a nitrosatable amine and residual nitrite. Artifactual formation with MOD and LTVD was demonstrated previously when 2,6-dimethylmorpholine was added to the sample before the distillations, and the *N*-nitroso derivative was detected by GC–TEA (8). Even though SFE and SPE were

Table 1. SFE recovery of 10 nitrosamines from a turkey bacon sample fortified at 10 ppb

N-Nitroso compound	Recovery, %			
	Range	Mean ($n = 12$)	SD	CV
NDMA	82.8–119.4	100.2	11.6	11.5
NMEA	91.2–101.5	96.6	3.1	3.2
NDEA	99.4–115.6	108.0	4.6	4.3
NDPA	97.5–111.6	103.4	3.8	3.7
NAZET	92.5–109.6	100.9	5.2	5.1
NDBA	78.1–102.7	87.3	6.5	7.4
NPIP	93.5–110.4	103.9	5.2	5.0
NPYR	99.1–110.5	104.1	4.0	3.8
NMOR	96.2–106.7	102.0	3.2	3.1
NHMI	95.4–108.3	102.2	3.5	3.4

Table 2. Determination of nitrosamines in bacon by 4 methods[a]

Sample	*N*-Nitrosopyrrolidine, ppb				*N*-Nitrosodimethylamine, ppb			
	SFE	SPE	MOD	LTVD	SFE	SPE	MOD	LTVD
1	4.0	3.8	4.1	3.4	0.6	0.0	0.5	0.7
2	6.5	6.1	7.4	6.7	0.9	1.2	0.6	0.4
3	1.4	1.4	1.8	1.0	0.9	ND[b]	0.4	0.6
4	3.4	3.0	3.4	3.2	0.9	0.8	ND	ND
5	2.4	1.1	3.3	2.7	ND	1.5	ND	0.5
6	10.9	10.1	11.2	11.0	1.3	1.9	1.8	1.7
7	4.9	4.4	6.4	6.7	1.6	1.1	ND	0.9
8	1.3	1.2	1.9	1.3	0.7	ND	ND	ND
9	9.5	8.9	10.5	9.5	0.9	1.4	0.8	0.6
10	6.2	5.0	6.1	5.5	ND	1.4	0.4	0.9
11	3.3	3.5	5.0	4.8	0.6	1.4	0.5	1.1
12	0.7	0.6	1.0	0.8	ND	1.3	ND	ND
13	1.7	1.4	2.3	2.1	1.5	1.3	ND	0.6
14	2.3	2.5	2.7	2.6	1.3	1.6	0.5	0.7
15	2.0	2.0	2.9	2.7	0.8	1.4	0.5	0.9
16	1.6	1.1	2.4	1.4	2.4	1.1	ND	0.9
17	20.2	18.8	24.6	32.1	1.4	1.2	1.7	1.6
18	6.4	5.2	8.3	8.3	0.7	1.0	0.7	0.6

[a] Average of duplicate determinations; corrected for recovery of internal NDPA standard.
[b] ND, none detected.

free of artifact formation, the methods were significantly different from each other. A previous comparison of these 2 methods for determination of NDBzA in ham (5) showed no significant difference. The difference this time with fried bacon may be due to more efficient extraction of NPYR with SFE. It is important, however, that the coefficient of variation (CV) for the SFE method for NPYR was the lowest of all the methods. NDMA results are also shown in Tables 2 and 3. Because of its volatility, as much as 75% of NDMA can be lost in the fumes during frying (19). As a result, even though there was a statistical difference among methods, the mean values were too low, about 1 ppb, to allow definite conclusions.

The finding of higher nitrosamine concentrations, especially of NPYR, in the bacon drippings suggests they are formed from precursor(s) in bacon adipose tissue (20, 21). Mottram et al. (22) found that fat produced 12 times the amount of NPYR and 6 times more NDMA than did the separated lean components. For this reason, SFE was also evaluated for its efficiency in extracting nitrosamines from the fried bacon drippings analyzed previously. Under the same SFE conditions used for fried bacon, the fat clogged the SFE restrictor tube. As with most SFE apparatus, the potential loss of analyte is greatest at the restrictor–collector interface (6). Changing the flow rate from 3.0 to 2.2 L/min eliminated the problem.

The SFE method was then checked for artifactual nitrosamine formation by addition of 1 and 5 ppm morpholine to some bacon drippings; no NMOR was detected. Average recoveries of bacon-specific nitrosamines by the SFE method from corn oil ($n = 6$) were 75.5% for NDMA, 81.6% for NPYR, and 83.4% for NDPA, the nitrosamine used as internal standard. The results obtained by SFE for 11 samples of drippings were compared with those obtained by 2 other methods: one, a published distillation procedure, and the other, a nondistillation modification of our SPE method for fried bacon. The results indicated that the SFE method extracted more nitrosamine than did the distillation method, in some cases almost double the

Table 3. Overall means and CVs of NPYR and NDMA determined in fried bacon by 4 methods

Method	*N*-Nitrosopyrrolidine, ppb[a]			*N*-Nitrosodimethylamine, ppb[a]		
	Mean	Group[b]	CV, %	Mean	Group	CV, %
SFE	4.9	A	4.1	0.9	A	12.6
SPE	4.5	B	6.3	1.1	B	7.9
MOD	5.9	C	9.1	0.4	C	18.9
LTVD	5.8	C	6.7	0.7	D	29.4

[a] $n = 18$.
[b] Means with different letters are significantly ($p < 0.05$) different from each other (Duncan's multiple range test).

Table 4. Determination of nitrosamines in bacon drippings by 3 methods[a]

Sample	*N*-Nitrosopyrrolidine, ppb			*N*-Nitrosodimethylamine, ppb		
	SFE	SPE	Distillation	SFE	SPE	Distillation
1	10.7	9.1	5.0	4.1	3.7	3.2
2	18.0	14.6	7.1	3.1	3.3	2.2
3	20.2	17.9	14.9	2.9	2.7	2.0
4	6.5	6.4	4.1	ND[b]	0.6	ND
5	9.8	9.1	4.5	3.7	3.6	2.9
6	11.2	9.6	4.9	5.0	4.5	3.6
7	12.0	9.5	5.3	2.2	2.1	1.5
8	14.0	12.8	7.3	3.6	3.6	3.1
9	8.6	8.3	4.7	3.1	3.4	2.3
10	13.4	11.8	6.7	3.1	2.5	1.9
11	12.1	11.6	6.2	2.5	2.0	1.5

[a] Average of duplicate determinations; corrected for recovery of internal NDPA standard.
[b] ND, none detected.

amount (Table 4). When these higher SFE values were observed, additional experiments were conducted to rule out artifactual formation. A few samples reanalyzed by both methods ensured that the values were correct. Results of a third method, SPE, were closer to the SFE values. The average recoveries of NDPA internal standard for the 3 methods were SFE, 74.9 ± 7.5%; distillation, 79.8 ± 8.3%; and SPE, 80.3 ± 6.0%. Statistical analysis of the results ($n = 11$) indicated a significant difference ($p < 0.05$) for both NPYR and NDMA among the methods. The mean values for NPYR (in ppb), were SFE, 12.4; distillation, 6.4; and SPE, 11.0. For NDMA, the means (in ppb) were SPE, 3.0; distillation, 2.3; and SPE, 2.8.

Conclusions

The SFE procedure reported here is a simple, rapid, solvent-sparing, and reproducible means for extracting NPYR from fried bacon and its drippings. It is not susceptible to artifactual nitrosamine formation, and it is superior to other methods for nitrosamine determination. The method, without GC–TEA, which is common to all procedures, takes less than 1 h, reducing analysis time for volatile nitrosamines. For fried bacon, each SFE analysis uses a total of 17 mL solvent compared with 125–475 mL used by the other methods. The unique properties of SC-CO_2, which include higher diffusivity combined with lower viscosity and temperature–pressure controlled density–solvent strength, make SFE an attractive alternative to conventional liquid solvent extraction techniques. Therefore, we recommend that SFE be used for the analysis of fried bacon and its drippings.

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